The endocannabinoid system in bull sperm and bovine oviductal epithelium: role of anandamide in sperm–oviduct interaction

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Abstract

Anandamide binds to cannabinoid receptors and plays several central and peripheral functions. The aim of this work was to study the possible role for this endocannabinoid in controlling sperm–oviduct interaction in mammals. We observed that bull sperm and bovine oviductal epithelial cells express cannabinoid receptors, CB1 and CB2, and fatty acid amide hydrolase, the enzyme that controls intracellular anandamide levels. A quantitative assay to determine whether anandamide was involved in bovine sperm–oviduct interaction was developed. \(\text{R}(\pm)\)-methanandamide, a non-hydrolysable anandamide analog, inhibited sperm binding to and induced sperm release from oviductal epithelia. Selective CB1 antagonists (SR141716A or AM251) completely blocked \(\text{R}(\pm)\)-methanandamide effects. However, SR144528, a selective CB2 antagonist, did not exert any effect, indicating that only CB1 was involved in \(\text{R}(\pm)\)-methanandamide effect. This effect was not caused by inhibition of the sperm progressive motility or by induction of the acrosome reaction. Overall, our findings indicate for the first time that the endocannabinoid system is present in bovine sperm and oviductal epithelium and that anandamide modulates the sperm–oviduct interaction, by inhibition of sperm binding and induction of sperm release from oviductal epithelial cells, probably by activating CB1 receptors.

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Introduction

The mammalian oviduct acts as a functional sperm reservoir providing a suitable environment that allows the maintenance of sperm fertilization competence until ovulation takes place (Harper 1994). After mating, mammalian spermatooza (SPZ) are sequestered in the lower oviduct through adhesion to the epithelial cells lining its lumen (Smith & Yanagimachi 1990). SPZ attachment to and release from oviductal reservoirs play a role not only in the temporal coordination of fertilization but also in assuring that an adequate number of SPZ reach the site of fertilization in a controlled way. This interaction is thought to prolong sperm life by delaying capacitation until ovulation-associated signals, such as progesterone or cathecolamines (Hunter 2008), induce the release of adhering sperm subpopulations (Smith & Yanagimachi 1991, Harper 1994). It has been reported that, in different species, particular sperm subpopulations are selected by cell–cell adhesion in in vitro cultures with oviductal cells. Particularly in bovines, in vitro selected sperm are characterized by fertilization competence (Gualtieri & Talevi 2003).

Although sperm adhesion to the oviductal epithelium is essential for successful fertilization, little is known about the molecules involved in its adhesion and release. However, there are some works that demonstrate the involvement of species-specific carbohydrate recognition in sperm–oviduct interaction (Lefebvre et al. 1997, Suarez 2001). Particularly in bovines this interaction involves fucose recognition (Lefebvre et al. 1997). In addition, Gwathmey et al. (2003) have demonstrated that bovine plasma seminal protein PDC-109 promotes bull sperm binding to oviduct epithelium in vitro and that it could be involved in the formation of the oviduct reservoir. Recently, Ignotz et al. (2007) found that annexins (that contain fucose residues) might be possible candidates for sperm receptors on bovine oviductal epithelium. Besides, it is known that sulfated glycoconjugates are powerful inducers of sperm release in vitro possibly by promoting sperm capacitation (Gualtieri et al. 2005) and/or disrupting the binding of bovine seminal plasma protein to annexins (Ignotz et al. 2007).

Anandamide (\(N\)-arachidonylethanolamide, AEA) is an endocannabinoid that activates specific cannabinoid
receptors (CB-R), CB1 and CB2, located on the surface of target cells (Howlett et al. 2002). AEA is synthesized from membrane phospholipid precursors by N-acylphosphatidylethanolamine–selective phospholipase D. Once released, AEA effect is quickly terminated by membrane-bound fatty acid amide hydrolase (FAAH; Di Marzo et al. 1994), suggesting a critical role for this lipid during cell signaling (Schuel 2006). CB-R are widely distributed in neuronal cells and in somatic cells from peripheral organs including the reproductive system (Paria et al. 2002, Wang et al. 2004, Maccarrone et al. 2005, Schuel & Burkman 2005). Significant levels of AEA are found in seminal plasma, mid-cycle oviductal fluid, and follicular fluid (Schuel et al. 2002b). Sperm are sequentially exposed to those levels of AEA as they swim from the ejaculate deposited in the vagina to the fertilization site in the oviductal ampulla (Yanagimachi 1994, Schuel et al. 2002b). Thus, AEA might regulate sperm capacitation and its fertilizing potential within the reproductive tract (Schuel et al. 2002a, 2002b, Rossato et al. 2005). Recently, it has been reported that human and boar sperm express CB1 and that CB1 activation modulates motility, capacitation, and acrosome reaction, events that take place in the oviduct (Maccarrone et al. 2005, Rossato et al. 2005). Consistently, boar sperm produces AEA (Maccarrone et al. 2005) and expresses FAAH and CB2. Studies in human and porcine sperm suggest the existence of an AEA gradient in the oviduct that might regulate sperm functions (Maccarrone et al. 2005, Schuel & Burkman 2005).

Since evidence indicates that AEA signaling might regulate sperm functions (Schuel et al. 2002a, Maccarrone et al. 2005, Rossato et al. 2005, Cobelli et al. 2006), which AEA is present in the mammalian oviduct (Schuel et al. 2002b, Wang et al. 2004) and that estradiol increases AEA synthesis (Maccarrone et al. 2002), we hypothesized that during the peri-ovulatory period, oviductal AEA might participate in sperm–epithelia crosstalk. Thus, our aims were 1) to characterize the endocannabinoid system in bull sperm and bovine oviductal epithelial cells (BOEC) and 2) to investigate the participation of AEA in sperm–oviduct interaction.

Results

Characterization of the endocannabinoid system

To study the participation of the endocannabinoid system in bovine sperm–oviduct interaction, we first investigated the expression of CB-R in both spermatozoa (SPZ) and oviductal epithelial cells (BOEC). As shown in Fig. 1A and B (upper panel) and Fig. 2A and B (upper panel), both bovine sperm and oviductal cells expressed CB1 and CB2 mRNA. Western blot analysis showed that in sperm cells and oviductal cells the specific CB1 antibody recognized a single band at ~58 kDa (Fig. 1A and 2A; lower panel), whereas the specific CB2 antibody reacted with a single band at ~38 kDa (Fig. 1B and Fig. 2B; lower panel). Immunoreactive specificity was assessed either by omitting the first antibody or by using the corresponding blocking peptide. CB1 antibody recognized one band at ~58 kDa in brain homogenates (used as CB1 positive control), which disappeared with the blocking peptide (data not shown).

To study the localization of CB1 and CB2 receptors, immunohistochemistry was performed on sperm and oviductal cells using specific primary antibodies. In bull SPZ, CB1 was mainly located on the head, specifically in the equatorial segment (strong stain) and the acrosomal region (patchy stain; Fig. 1C), whereas

Figure 1 Expression and localization of CB-R in bull sperm (SPZ). (A) CB1 and (B) CB2 mRNA (upper panel), and protein (lower panel) expression (n=3). B, bovine brain (CB1 positive control), S, bovine spleen (CB2 positive control), SPZ(–), incubation with the blocking peptide. (C) CB-R localization (scale bar=20 µm). Arrows indicate CB-R localization.

Figure 2 Expression and localization of CB-R in bovine oviductal epithelial cells (BOEC). (A) CB1 and (B) CB2 mRNA (upper panel) and protein (lower panel) expression (n=3). B, bovine brain (CB1 positive control), S, bovine spleen (CB2 positive control), BOEC(–), incubation with the blocking peptide. (C) CB-R localization (scale bar=20 µm).
CB2 was expressed in the apical region of the acrosome, in the post-acrosomal region of the head and in the principal piece of the tail (Fig. 1C). In epithelial oviductal cells, both CB1 and CB2 were mainly expressed in the plasma membrane (Fig. 2C). Cells incubated with the corresponding blocking peptide or without the primary antibody did not show non-specific stain (Figs 1C and 2C, Control).

FAAH protein content was then investigated by Western blotting. A comparative analysis between SPZ and BOEC indicated the presence of a single band with a molecular size of ~55 kDa for SPZ and of ~52 kDa for BOEC (Fig. 3). When lanes containing SPZ (Fig. 3) or BOEC (data not shown) were incubated without the primary antibody, FAAH band did not appear. Since FAAH has been previously detected in human placenta (Park et al. 2003), this tissue was used as a positive control (data not shown).

**AEA effect on sperm–oviduct interaction**

**Experiment 1 (co-incubations)**

Since our results indicated that SPZ and the oviductal epithelium expressed CB-R and FAAH and previous results suggested that an oviductal AEA gradient could be modulating sperm functions (Maccarrone et al. 2005, Schuel 2006), the next step was to investigate whether AEA participates in sperm–oviduct interaction. We therefore co-incubated BOEC with 0.5 × 10⁶ SPZ/ml and nanomolar concentrations of CB-R agonists because at mid-cycle AEA is present in human oviductal fluid at nanomolar concentrations (Schuel et al. 2002b) and because it was the lowest agonist concentration that had an effect (see Materials and Methods). We found that both Met-AEA (1.4 × 10⁻⁹ M) and AEA (10⁻⁹ M) significantly decreased the number of SPZ bound to BOEC by 35–40% when compared with control values (Fig. 4A). The following experiments were performed with Met-AEA as CB-R agonist. Then, we analyzed if an increase in endogenous AEA could produce a similar effect. For this purpose, co-cultures were incubated with URB597, a selective inhibitor of FAAH (Piomelli et al. 2006), the enzyme that degrades AEA and regulates its levels. We found that the incubation with URB597 (0.5 × 10⁻⁹ M), significantly decreased the number of SPZ bound to BOEC when compared with the control treatment (Fig. 4B). Then, in order to investigate the participation of CB-R in this interaction, we incubated BOEC and SPZ with different concentrations of CB1 or CB2 antagonists. Met-AEA (1.4 × 10⁻⁹ M) effect was completely reversed by the incubation with the selective CB1 antagonist, SR141716A (10⁻¹⁰ M; Fig. 5A). Moreover, SR144528, a selective CB2 antagonist, did not reverse the effect elicited by Met-AEA (Fig. 5B). In this experiment, we used different CB2 antagonist concentrations because 10⁻¹⁰ M did not reverse Met-AEA effect. The results indicated that neither 10⁻⁹ M nor 10⁻⁷ M of SR144528 was able...
Motility assessment

As previous works have indicated that AEA at \(10^{-7}\) and \(10^{-6}\) M inhibits sperm motility (Rossato et al. 2005, Cobellis et al. 2006), we investigated whether AEA or Met-AEA diminished the number of SPZ bound to BOEC as a result of a reduction in sperm motility. In order to address this issue, we measured bovine sperm progressive motility (PM) and vigor in the presence of nanomolar concentrations of Met-AEA, AEA and URB597. The results (Table 1) indicated that neither PM nor vigor was affected by Met-AEA (\(1.4\times10^{-9}\) M), AEA (\(10^{-9}\) M) or URB597 (\(0.5\times10^{-9}\) M).

Based on the results obtained in experiment 1, we hypothesized that Met-AEA could decrease bound sperm by either inhibiting their binding or triggering their release from BOEC. To test this, we designed two types of experiments: pre-incubation of either BOEC or SPZ with Met-AEA to analyze binding inhibition (experiments 2 and 3) and incubation of the co-cultures with Met-AEA to assess releasing effects (experiment 4).

Experiment 2 (BOEC pre-treatment)

This experiment was performed in order to investigate the effect of pre-treatment of BOEC with Met-AEA (\(1.4\times10^{-10}\) and \(1.4\times10^{-9}\) M). We observed that Met-AEA significantly inhibited SPZ binding to BOEC at \(1.4\times10^{-9}\) M but not at \(1.4\times10^{-10}\) (Fig. 6A). In addition, CB1 antagonist, SR1141716A, completely reversed the effect of Met-AEA at \(1.4\times10^{-9}\) M.

Experiment 3 (SPZ pre-treatment)

The next step was to study whether sperm pre-treatment with the CB-R agonist affected sperm binding. Data showed that sperm pre-treatment with Met-AEA at \(1.4\times10^{-9}\) M produced a significant inhibition when compared with control incubated in BSA-free sperm-TALP alone (Fig. 6B). Met-AEA effect was completely reversed by the incubation with the selective CB1 antagonist, SR1141716A (\(10^{-10}\) M). Interestingly, sperm pre-treatment with Met-AEA at \(1.4\times10^{-10}\) M did not have any effect (Fig. 6B), suggesting that SPZ can bind to BOEC when AEA concentrations are lower than nanomolar.

Table 1 Effect of cannabinoid agonists methanandamide or anandamide (Met-AEA or AEA) and of a fatty acid amide hydrolase (FAAH) inhibitor (URB597) on bovine sperm motility parameters: vigor and progressive motility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PM</th>
<th>T=0</th>
<th>Vigor</th>
<th>T=15</th>
<th>Vigor</th>
<th>T=60</th>
<th>Vigor</th>
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<tbody>
<tr>
<td>Control</td>
<td>76.6 ± 4.4</td>
<td>4.2 ± 0.4</td>
<td>66.3 ± 4.8</td>
<td>4.0 ± 0.1</td>
<td>62.9 ± 13.3</td>
<td>3.8 ± 0.4</td>
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<tr>
<td>AEA 10^{-9} M</td>
<td>76.7 ± 5.8</td>
<td>4.7 ± 0.6</td>
<td>73.3 ± 11.6</td>
<td>4.7 ± 0.6</td>
<td>65.0 ± 8.7</td>
<td>4.0 ± 0.1</td>
<td></td>
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<tr>
<td>Met-AEA 1.4×10^{-9} M</td>
<td>75.8 ± 5.9</td>
<td>4.2 ± 0.4</td>
<td>63.7 ± 1.9</td>
<td>3.7 ± 0.5</td>
<td>61.2 ± 6.5</td>
<td>3.6 ± 0.5</td>
<td></td>
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<tr>
<td>URB597 0.5×10^{-9} M</td>
<td>74.8 ± 7.4</td>
<td>4.4 ± 0.6</td>
<td>69.6 ± 5.5</td>
<td>3.8 ± 0.5</td>
<td>61.6 ± 6.5</td>
<td>3.6 ± 0.6</td>
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PM results are expressed as percentage (mean ± s.d.). Arithmetic means of the subjectively assessed motility parameters. Vigor results are expressed in arbitrary units between 0 and 5. T = 0, 15, 60 min: time of incubation of initial sperm suspensions in BSA-free sperm-TALP alone (control) or with the drugs.


Figure 5 (Experiment 1). Effect of CB-R antagonists on sperm–oviduct interaction. Oviductal monolayers were co-incubated for 1 h at 39°C with 0.5 × 10⁵ sperm/ml and BSA-free sperm-TALP alone (control) or Met-AEA (A and B) plus SR141716A (A, CB1 antagonist) or plus SR144528 (B, CB2 antagonist). Bars indicate the number of spermatozoa bound to the monolayers. Data are expressed as percentage (%) of bound spermatozoa relative to control and represent the mean ± s.e.m. of bound sperm/0.11 mm² monolayer (A, n = 6 B, n = 5), a ≠ b, P < 0.01.
Analysis of data regarding the effect of Met-AEA on the release of bound sperm from BOEC showed that Met-AEA induced a significant sperm release at $1.4 \times 10^{-9}$ M, but that Met-AEA at $1.4 \times 10^{-9}$ M did not have effect (Fig. 7). In addition, Met-AEA induced sperm release from BOEC was significantly reversed by CB1 antagonist, SR141716A ($10^{-10}$ M; Fig. 7).

**Assessment of SPZ acrosomal status**

In our model, it is possible that bound sperm could be detached in response to the acrosome reaction. In order to investigate this possibility, we analyzed sperm acrosome integrity of different sperm subpopulations from experiment 4: 1) sperm adhered to BOEC after treatment with Met-AEA ($1.4 \times 10^{-9}$ M) and 2) sperm collected after induction of release by addition of Met-AEA ($1.4 \times 10^{-9}$ M). Results indicated that most of the sperm from both subpopulations presented intact acrosomes (SPZ bound to BOEC: $95.0 \pm 2.9\%$ of SPZ with intact acrosome/total bound sperm; SPZ released from BOEC: $82.0 \pm 6.6\%$ sperm with intact acrosome/total released sperm). Figure 8 shows sperm with intact acrosomes bound to BOEC both in control conditions and with Met-AEA at nanomolar concentrations.

**Discussion**

Evidence suggests that endocannabinoid signaling acts in the reproductive tract of mammals (Taylor et al. 2007). In the present work, we demonstrated for the first time...
that both bovine sperm and oviductal epithelial cells express CB-R, CB1 and CB2 and that AEA at physiological concentrations modulates sperm–oviduct interaction.

Our results indicate that CB1 and CB2 are expressed in bovine SPZ, as demonstrated by the presence of both the mRNA and the protein corresponding to these receptors. Moreover, CB1 is localized in the equatorial segment and, intermittently, in the acrosome, and CB2 is located mainly in the apical region of the acrosome, in the post-acrosomal region of the head and in the principal piece of the tail. The fact that, in sperm, CB1 and CB2 receptors showed a different localization suggests that once AEA binds to them, they might activate selective transduction pathways during the fertilization process. The CB-R localization shown in this work supports possible roles of these receptors in modulating sperm functions such as acrosomal status and motility. Sperm CB-R expression has been also reported in other mammalian species. Maccarrone et al. (2005) have described the presence of CB1 and CB2 in boar sperm; however, they found CB1 mainly localized in the post-acrosomal region and the tail. Rossiato et al. (2005) detected only CB1 in human sperm and found its localization to be confined to the head. On the other hand, we found that both CB-R are expressed in oviductal epithelia, and that they are mainly localized in the plasma membrane. Previously, Wang et al. (2004) have described the presence of CB1 receptor, but not CB2, in the muscular layer of the mouse oviduct. In addition, several authors have found that, in mice, both CB1 and CB2 receptor subtypes are expressed in preimplantation embryos, whereas only CB1 is expressed in the oviduct and uterus (Das et al. 1995, Paria et al. 1995, 2001, Wang et al. 2004). To our knowledge, this is the first report that demonstrates CB2 expression in oviductal epithelia.

In the present work, we also found that FAAH, one of the enzymes that regulate AEA endogenous levels, is expressed in bull sperm and in bovine oviductal epithelium. We found a protein band within the range of molecular weights previously described for this enzyme (Cobellis et al. 2006, Wei et al. 2006). Consistently with our results, isolated immature Sertoli cells (Maccarrone et al. 2003), boar sperm (Maccarrone et al. 2005), germ cells (Cobellis et al. 2006), and mouse oviductal epithelium (Wang et al. 2006) have also been shown to express FAAH.

Some studies suggest the existence of an AEA concentration gradient in the oviduct (Schuel 2006). Wang et al. (2006) have demonstrated in mice that a critical balance between AEA synthesis and degradation creates locally an appropriate ‘anandamide tone’ required for normal embryo development, oviductal transport, implantation, and pregnancy. Besides, it has been shown that during their ascent in the female reproductive tract, mammalian sperm transiently adhere to epithelial cells lining the fallopian tube until signals related to ovulation release fertile sperm within the oviduct (Harper 1994, Hunter 2008). Many evidence exist about the role of adhesion molecules and oviductal secretions in the maintenance of the sperm life span (Pollard et al. 1991, Abe et al. 1995, Lefebvre et al. 1997, Smith & Nothnick 1997). In addition, the mechanisms involved in fertile sperm release have been studied. Lefebvre et al. (1997) found that bovine sperm binding to oviductal epithelium involves fucosa recognition and that fucosidase treatment produces reduction of sperm binding suggesting that the functional ligand is in the epithelium, while lectin is on the sperm. In addition, recently Ignotz et al. (2007) found that annexins (that contain fucose residues) might be possible candidates for sperm receptors on bovine oviductal epithelium. The release of sperm adhering to oviductal epithelial cells in vitro is induced by sulfated glycoconjugates (Talevi & Gualtieri 2001) possibly by promoting sperm capacita
tion (Gualtieri et al. 2005) and/or by disrupting the binding of bovine seminal sperm proteins to annexins (Ignotz et al. 2007).

In the present study, CB-R agonists were tested for their ability to regulate sperm–oviduct interaction. We demonstrated that AEA and its stable analog Met-AEA, at nanomolar concentrations, decreased sperm binding to bovine oviductal cells in vitro. In addition, when FAAH was inhibited by URB597, a similar effect was observed, reinforcing the notion that an increase in endogenous AEA might decrease sperm binding to the oviductal epithelium. We were also interested in studying if CB-R, CB1 and CB2 were involved in the effects exerted by Met-AEA. We observed that antagonists of CB1, but not of CB2, reversed Met-AEA effect on sperm–oviduct interaction. Thus, it could be proposed that the decrease in sperm binding to oviductal epithelium caused by AEA in vivo is mediated by CB1 receptors.

It is well known that only motile sperm bind to the apical surface of oviductal epithelia (Thomas et al. 1994, Suarez 2002). Since we observed that AEA decreased sperm binding to BOEC, we decided to evaluate whether AEA modulated vigor and sperm PM, two well-known parameters used to describe bovine sperm motility. Results showed that incubations with Met-AEA, AEA or URB597 did not modify these parameters. Thus, the effect of anandamide on SPZ binding to BOEC was not due to a reduction in sperm motility. On the other hand, Rossiato et al. (2005) and Cobellis et al. (2006) have found that AEA at 10^{-7} and 10^{-6} M inhibits human and frog sperm motility respectively. Differences from our results could be explained based on the fact that those authors worked with other species or due to the concentrations of AEA assayed. In this work, the AEA concentration was tested within the physiological range (Schuel 2002b).

We demonstrated for the first time that Met-AEA, an AEA analog, inhibited sperm binding to oviductal cells at nanomolar concentrations but not at lower
concentrations, and, more interestingly, that it was capable of triggering the mechanism of sperm release. This observation suggests that AEA, at low levels, might allow the sperm to bind to the oviductal epithelium. However, when the level of AEA is increased, it might trigger the signals that stimulate sperm release. Talevi & Gualtieri (2001) demonstrated similar effects in bovine sperm, such as inhibition of sperm binding and the subsequent induction of release triggered by heparin and other sulfated glycoconjugates, known capacitative agents.

Data from pre-treatment experiments showing that Met-AEA effect was stronger on sperm rather than on oviductal cells suggest that AEA might induce sperm surface remodeling, thus modifying the molecules involved in sperm–oviduct interaction. However, when both types of cells were pre-treated with Met-AEA in binding inhibition experiments, the effect was completely reversed by CB1 antagonist SR141716A, indicating that the agonist effect was receptor selective. Thus, CB1 present both in sperm and oviductal cells could be participating in this effect in vivo. For this reason, we could not discard the possibility that AEA also induces surface remodeling on oviductal cell.

Other authors have found that, besides CB1 and CB2, endocannabinoid signaling through TRPV1 receptors could also influence several reproductive functions (Maccarrone et al. 2005). Although Met-AEA effect was significantly reversed by CB1 antagonist SR141716A, we do not discard the possible participation of other receptors, i.e., vanilloid receptor TRPV1, in sperm–oviduct interaction. Particularly, it has been published that CB1, TRPV1, and calcium seem to be in the same signaling pathway in some biological systems (Ross 2003). New experiments are being performed in order to elucidate whether TRPV1 is involved in this process.

The analysis of the acrosomal status in bound and released sperm populations after Met-AEA treatment did not reveal a significant increase in the percentage of acrosome-reacted sperm. Thus, this result indicates that the release of bound sperm did not involve the acrosome reaction, and is in agreement with data from Gualtieri & Talevi (2000), who observed that the addition of molecules capable of triggering sperm release from co-cultures causes a massive release of acrosome-intact sperm.

Fertilization in vivo requires that a proper number of sperm reach the oviductal ampulla in an appropriate physiological milieu, and should coincide with a viable unfertilized egg. Mammalian sperm penetrate the cervical mucus and enter the upper female reproductive tract and may be stored in reservoirs located in the cervix and oviductal isthmus. Some authors propose that hyperactivation and sperm capacitation, induced by unknown signals, may enhance the ability of sperm to release them from the oviductal epithelium (Dobrinski et al. 1997, Bosch et al. 2001). Conditioned media from whole oviduct (Anderson & Killian 1994) or from monolayers (Chian & Sirard 1995, Chian et al. 1995) have a capacitating activity that peaks at estrus and declines during the luteal phase. There is also evidence that AEA is present at mid-cycle in human oviductal fluids (Schuel et al. 2002b). In addition, recently we determined AEA synthesizing capacity in the bovine oviduct as previously was described in the mouse and rat uterus (Paria et al. 1996, Ribeiro et al. 2008). In this sense, we found that the ampulla showed higher activity compared with the isthmus (data not shown). These results support our speculation that changes in AEA levels during the estrous cycle might regulate fertilization-competent sperm release from oviductal reservoirs, thus allowing their ascent to the fertilization site. Based on our results and previous reports (Schuel 2006), we propose that an AEA gradient created during the peri-ovulatory period might be one of the signals that participate in the fertilization process promoting sperm capacitation and/or hyperactivation thus enhancing the ability of sperm to be release from oviductal reservoirs. Another possibility is that AEA, as a second messenger, might mediate the effect of other molecules, such as sulfated glycoconjugates, in the oviductal fluid and be involved in sperm releasing signaling pathways at the time of fertilization. To date, it has been shown that sulfated glycoconjugates (Bosch et al. 2001, Talevi & Gualtieri 2001) and cumulus–oocyte complexes (Brussow et al. 2006) also promote sperm release from oviductal reservoirs and that changes in oviduct mucosa do not participate in this mechanism (Lefebvre et al. 1995).

The mechanisms underlying AEA participation in sperm–oviduct interaction have not yet been elucidated, although the regulation of ion channels, adenylate cyclase activity and protein phosphorylation might be involved. The activation of the CB1 receptor in various cell types has been coupled to ion channel modulation (Di Marzo et al. 2002). Based on preliminary data in sperm (Rossato et al. 2005), it is possible that AEA induces rapid membrane hyperpolarization by activating potassium effluxes (K+) from sperm cytoplasm. Another possibility is that AEA might produce an increase in sperm calcium concentration (Ca2+), activate sperm capacitation and/or hyperactivation, and thus promote sperm release from oviductal reservoirs.

In vitro, sperm adhesion to the fallopian tube is beneficial for sperm fertile half-life and also allows the selection of higher quality sperm. Several works have reported that the oviduct selects those SPZ with intact acrosomes, uncapacitated status, superior morphology, and normal chromatin structure (Thomas et al. 1994, Lefebvre & Suarez 1996, Ellington et al. 1999, Gualtieri & Talevi 2000). The study of the molecules involved in the regulation of sperm–oviduct adhesion and release in an in vitro model may provide new basic information about early reproductive events, allowing the
development of alternative methods for a more physiological sperm selection in assisted reproductive technologies.

In summary, in the present study we demonstrated for the first time that AEA is a modulator of sperm–oviduct adhesion in vitro. These results are supported by the fact that both bull SPZ and the bovine oviduct express receptors for endocannabinoids and that AEA might act through CB1. Further investigations should be performed to elucidate whether AEA is capable of activating sperm capacitation or hyperactivation, inducing sperm release from oviductal reservoir and enhancing sperm fertilization ability. It is also necessary to understand which mechanisms regulate AEA secretion and that molecules are involved in AEA pathway.

Materials and Methods

Chemicals

R(+)-methanandamide, anandamide, luminol, and p-coumaric acid (Fluka) were purchased from Sigma Chemical Company. Glass wool to perform columns was obtained from Micro-Fibre Manville (Denver, CO, USA). All other chemicals were of analytical grade. M199 medium, gentamicin and fungizone were obtained from Gibco (Invitrogen). AM-251 (Gatley et al. 1996) was from TOCRIS. SR141716A (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide; Rinaldi-Carmona et al. 1994) and SR144528 (N-[1S]-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4 chloro-3-methylphenyl)-1-(4-methyl-benzyl)-pyrazole-3 carboxamide; Rinaldi-Carmona et al. 1998) were kind gifts from Sanofi-Aventis Recherche (Montpellier, France). Polyclonal rabbit antibody against CB1, which is known to cross react with different species such as mouse, rat, monkey, human, Xenopus, and chick (Tsou et al. 1998, Straiker et al. 1999, Cobellis et al. 2006), was kindly provided by Dr Kenneth Mackie. Bovine CB1 receptor possesses about 72% identity of sequence compared with rat and mouse. Polyclonal rabbit antibody against FAAH, which is known to cross react with different species such as rat, human, and mouse, was kindly provided by Dr Cravatt (Wiang et al. 2006). FAAH enzyme is well conserved in its primary structure. Bovine FAAH possesses a range of 75, 78, and 80% identity of sequence compared with mouse, rat, and human respectively. Polyclonal rabbit antibody against CB2, which is known to cross react with human and rat, was from Affinity BioReagents (Golden, CO, USA Jiang et al. 2007). CB2 bovine sequence has not yet been published. Zoratti et al. (2003) used primers homologous to the human and mouse sequences for CB2 amplified the bovine CB2 receptor from bovine spleen, as this tissue is supposed to have a high expression level of this receptor. Alexa-Fluor555 goat anti-rabbit IgG and Alexa-Fluor594 donkey anti-mouse IgG were from Molecular Probes (Invitrogen), and URB597 (a selective FAAH inhibitor) was from Cayman Chemical Company (Migliore Laclaustra, Argentina).

Six-well tissue culture dishes were from NUNC (Lobov) and glass wool columns for sperm selection from Micro-Fibre Manville.

Media cultures

M199 medium supplemented with 50 μg/ml gentamicin, 1 μg/ml fungizone and 10% (v/v) FCS was used for oviductal incubations and development of monolayer cultures (Gualtieri & Talevi 2000). Sperm handling and co-culture experiments were performed with Tyrode bicarbonate buffered medium (sperm-TALP, Parrish et al. 1988) without BSA (BSA-free sperm-TALP medium). BSA was omitted because it can bind to endocannabinoids and thus inhibit AEA uptake (Di Marzo et al. 1994, De Petrocellis et al. 2001). The SPZ viability was not affected without BSA because the gametes were washed and immediately co-incubated with oviducal cells.

Sperm preparation

Frozen bovine semen from five bulls (20–25 × 10⁶ SPZ/0.5 ml straw), obtained from CIALE (Artificial Insemination Center, Buenos Aires, Argentina) was used. Straws were thawed in a water bath (37 °C for 30 s). SPZ were subjected to sperm selection using glass wool columns (Calvo et al. 1989) and washed by centrifugation at 800 × g with BSA-free sperm-TALP. Pellets were assessed for sperm concentration and motility using a hemocytometer mounted on a microscope stage heated at 38 °C.

Subjective analysis of sperm motility

Sperm samples were rated on the basis of dynamic parameter data (PM and vigor) that were evaluated under a light microscope (38 °C, magnification 300×) using a hemocytometer. Samples were evaluated, thrice by the same observer, in the following cases: immediately after washing (T=0) and after 15 and 60 min of incubation (control and treatments). At least 200 SPZ were counted in each sample.

Sperm motility was estimated placing a sample of diluted semen on a microscope slide, examining it with a microscope and estimating the fraction of the population that is motile. More specifically, a sample of semen was diluted in warm extender or buffered saline, and about 10 μl of this sample was pipetted onto a clean, pre-warmed slide under a light microscope at 38 °C. At least 10 widely spaced fields were examined to provide an estimate of the percentage of motile cells. Sperm motility was assumed to be the percentage of sperm that are progressively motile. A progressively motile sperm swims briskly forward in a relatively straight line, as opposed to moving in circles. PM was rated from 0 to 100%. Results are expressed as percentage± s.d. of motile SPZ/total SPZ.

Vigor (sperm motility status or quality of motility) was scored on a scale from 0 (without movement) to 5 (fast progressive movement).

Samples presenting at least 70% average of PM and vigor ≥ 4 were considered suitable for experiments.

Oviducal cell cultures

Oviducts were collected at the time of slaughter, transported at 4 °C, cleaned off surrounding tissues and washed thrice.
in sterile PBS at 4 °C. Then, they were cut, flushed with sterile PBS and squeezed by pressure with tweezers. Laminae of BOEC were recovered from different animals, selected on the basis of ciliary beating, and pooled together. Immunocytochemistry staining was performed to confirm > 90% epithelial cell content in the pools. BOEC were washed by centrifugation at 1500 g for 5 min and incubated in M199 medium at 39 °C in a 5% CO2 atmosphere. Incubations were performed in six-well tissue culture dishes with 12 mm round cover slips on the well bottom. After 48 h, BOEC were washed by centrifugation (1500 g for 5 min) and seeded again in tissue dishes. M199 medium was changed every 48 h. Once cell confluence was attained, oviductal monolayers from the same pool of animals were washed thrice in BSA-free sperm-TALP and left in this medium for 90 min until sperm addition.

**CB1 and CB2 RT-PCR**

Total RNA was isolated from bovine oviducts and SPZ using Trizol reagent (Invitrogen), according to the manufacturer’s recommendations. RNA concentration was determined by spectrophotometry measuring the absorbance at 260 nm. cDNA amplifications and PCR were performed as previously described (Das et al. 1995). First-strand cDNA was synthesized from 1 μg total RNA, using Maloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT) and random primers (Invitrogen), according to the manufacturer’s instructions in the presence of recombinant RNase inhibitor. After first-strand synthesis, PCR was performed with the following oligonucleotide primers (Das et al. 1995): CB1 transcript: 5’-GAGGAAACATCCAGTGTTGCGG-3’ (sense), 5’-CATTGGGGCTGTCTTACGG-3’ (antisense); CB2 transcript: 5’-CCTGTGTTGAAAGATCGGACACG-3’ (sense), and 5’-GGTACGAGATCAGCGGCG-3’ (antisense) and Invitrogen Custom Primers). Amplifications were performed using Taq DNA polymerase enzyme (Invitrogen).

PCR was performed as follows: 94 °C for 3 min (initial denaturation) and 39 cycles at 94 °C for 1 min, 55.5 °C for 30 s, and 72 °C for 2 min. PCR products were separated on a 2% (w/v) agarose gel, stained with ethidium bromide and recorded under u.v. light with a digital camera (Olympus C5060).

**CB-R and FAAH identification by western blotting**

CB1, CB2, and FAAH protein expression were determined by western blotting analysis using specific antibodies. Oviductal cells or bovine SPZ were incubated in triple detergent buffer (PBS (pH 7.4) with 0.02% w/v sodium azide, 0.1% w/v SDS, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate) containing 10 μg/ml leupeptin, 2 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 1 mM EDTA, 1 mg/ml benzamidine, 10 μg/ml dithiothreitol, 1 mg/ml caprylic acid, and 1 mM sodium orthovanadate. Cells were sonicated and centrifuged for 10 min at 20000 g. Protein determination was assayed by the Bradford method (Bradford 1976) using BSA as standard. Sperm (100 μg/lane) or oviduct (70 μg/lane) proteins were separated in 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membrane non-specific binding sites were blocked and incubated with primary CB1, CB2, or FAAH (1:300 in 5% w/v dried fat milk) antibodies followed by incubations with goat anti-rabbit HRP-conjugated IgG. Homogenates from bovine brain (B) and spleen (S) were used as positive controls for CB1 and CB2 respectively. Immunoreactive specificity was assessed by either the corresponding blocking peptide or omitting the first antibody. Bands were visualized using chemiluminescence detection reagents and exposed to Kodak X-OMAT films.

**Localization of CB-R in bovine sperm and oviductal cells**

Cells (SPZ and BOEC) were fixed (5 min, at room temperature (RT), 0.2% w/v paraformaldehyde), immobilized on slides and permeabilized with cold methanol. Non-specific binding sites were blocked (60 min, 40 mg/ml BSA–PBS). Slices were treated with primary CB1 or CB2 antibodies (1:50) and incubated afterwards with Alexa555-conjugated anti-rabbit IgG (1:2000). Immunoreactive specificity was assessed by either omitting the first antibody or the blocking peptide. Sperm cells were mounted and examined under a fluorescence microscope (Olympus IMT2) coupled to a digital camera. Oviductal cells were also examined under a confocal laser imaging system (Nikon C1; Plan Apo 40×/0.95).

**AEAA participation in sperm–oviduct interaction: experimental design**

Within each experiment, confluent BOEC monolayers from pooled oviducts were inseminated with the same sperm suspension (0.5-1×10^6 sperm/ml of BSA-free sperm-TALP/well) for 60 min at 39 °C in a 5% CO2 atmosphere.

At the end of the co-cultures, unbound sperm populations were removed by washing thrice with fresh medium. Oviductal monolayers with attached sperm were fixed as described below.

Experiment 1 was performed to investigate AEA effect on sperm–oviduct interactions. First, BOEC were co-incubated with 0.5×10^8 sperm/ml and increasing concentrations of the CB-R agonist Met-AEA (1.4×10^−10 to 1.4×10^−6 M). Except for 1.4×10^−10 M, all agonist concentrations had an effect on sperm–oviduct interaction (data not shown). To perform the experiments, 1.4×10^−9 M of Met-AEA was chosen because it was the lowest concentration able to modulate sperm–BOEC interaction. Monolayers were incubated with R(+)-methanandamide (Met-AEA, 1.4×10^−9 M), (Met-AEA, 1.4×10^−9 M), a selective FAAH inhibitor URB597 (0.5×10^−9 M, Kathuria et al. 2003) and/or CB-R selective antagonists (CB1: SR141716A (10^−7 M); or AM251(10^−7 M)) and simultaneously inseminated with 1×10^6 sperm/ml BSA-free sperm-TALP. After 60 min, unbound SPZ were removed and monolayers were fixed and analyzed as described below.

Since drugs were dissolved in ethanol or DMSO, control incubations were performed with the same final vehicle concentrations (0.0001% v/v). Based on experiment 1 results, we performed experiments 2, 3 and 4.
In experiment 2, we studied if pre-treatment of BOEC with Met-AEA was able to influence sperm binding. Oviductal monolayers were pre-treated for 15 min with Met-AEA (1.4×10^{-10} and 1.4×10^{-9} M) or Met-AEA (1.4×10^{-10} M) plus SR141716A (10^{-10} M), washed and co-cultured with 0.5×10^6 sperm/ml for 60 min. Unbound sperm were washed and monolayers were fixed for quantitative analysis.

In experiment 3, we studied whether pre-treatment of sperm with Met-AEA influenced sperm binding to BOEC. Sperm were pre-incubated for 15 min with Met-AEA (1.4×10^{-10} and 1.4×10^{-9} M) or Met-AEA (1.4×10^{-10} M) plus SR141716A (10^{-10} M), washed in BSA-free sperm-TALP by centrifugation at 800 g for 5 min and co-cultured with BOEC at 0.5×10^6 sperm/ml for 60 min. Control sperm were pre-incubated with BSA-free sperm-TALP alone, washed and co-cultured as above. Unbound sperm were washed and monolayers were fixed for quantitative analysis.

In experiment 4, we assessed the effect of Met-AEA on sperm release from oviductal monolayers. Wells containing BOEC monolayers were inseminated with 0.5×10^6 sperm/ml for 60 min and afterwards unbound sperm were washed. Then, Met-AEA (1.4×10^{-10} and 1.4×10^{-9} M) or Met-AEA (1.4×10^{-10} M) plus SR141716A (10^{-10} M) were added for 15 min. Control and treated wells were washed to remove unbound sperm and fixed for quantitative analysis.

**Quantitation of bound sperm**

After co-cultures, oviductal monolayers with adhering SPZ were fixed in glutaraldehyde 2.5% v/v for 60 min at RT, extensively washed and mounted on a glass slide. The number of bound sperm was determined by analyzing 20 fields of 0.11 mm^2/cover slip under a phase contrast microscope (300×).

Results are expressed as % SPZ bound to BOEC as compared with the control value. It was assumed that the controls in each experiment presented 100% of SPZ bound to BOEC. The results are expressed in percentages because in different pools of BOEC under control conditions the number of bound sperm was very variable.

Oviductal epithelium from 6 to 8 cows was pooled. Each pool was divided into control and treated cultures. The range of sperm bound/0.11 mm^2 BOEC in control conditions was:

- Experiment 1: 45–100 bound sperm.
- Experiment 2: BOEC pre-treated: 30–100 bound sperm.
- Experiment 3: sperm pre-treated: 10–30 bound sperm.
- Experiment 4: 20–50 bound sperm.

**Assessment of acrosomal status of bound and released sperm**

Study of acrosomal status of bound and released sperm was performed on the following sperm subpopulations: 1) sperm adhered to BOEC monolayer cultures after treatment with Met-AEA (1.4×10^{-9} M) or BSA-free sperm-TALP alone (control) and 2) bound sperm collected at 15 min after induction of release by addition of Met-AEA (1.4×10^{-9} M). Samples were washed and fixed in 0.2% w/v paraformaldehyde in PBS for 1 h at RT. For determination of the acrosomal status, cells were washed in PBS and permeabilized in cold methanol for 5 min at 20 °C, washed in PBS and incubated for 60 min at RT with 10 μg/ml *Pismus sativum* agglutinin conjugated with FITC (PSA–FITC). Finally, samples were washed, mounted in glycerol: PBS and observed at a fluorescence microscope. A total of 100 sperm were assessed for each sample. Sperm with no fluorescence over the acrosomal region and sperm with a thin band of green fluorescence over the equatorial region of the sperm head were scored as acrosome-reacted sperm (Cross & Meizel 1989). In addition, sperm were scored as acrosome-reacted when the smooth acrosomal border was replaced by a wrinkled border as described by Galantino-Homer et al. (1997).

**Statistical analysis**

Data were analyzed by GLM procedures of one-way ANOVA (STATISTICA 6.0 software, StatSoft Inc., Tulsa, OK, USA, 1984-2001). Raw data were analyzed by Cochran, Hartley and Bartlett test and GLM procedures were applied in all variance analyses. Data that did not fulfill assumptions were modified by the arcsine transformation to be normalized. Pairwise comparisons of means were made with Tukey or Fisher honestly significant differences. Results were expressed as mean±S.E.M of at least three independent determinations. Differences were considered to be significant when *P*<0.05 or less.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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